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CHEMOTACTIC AND GROWTH RESPONSES OF MARINE BACTERIA TO ALGAL EXTRACELLULAR PRODUCTS

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It has been known for many years that filtrates from axenic algal cultures may be enriched with organic compounds. These materials, including simple amino acids and peptides, sugars, polyalcohols, and occasionally vitamins, enzymes, and toxins, are usually lumped under the term "extracellular products" (Fogg, 1966). Studies using natural populations of phytoplankton have shown that extracellular products are not mere laboratory artifacts, and that, depending upon environmental conditions, they account for 1–20% of the total photoassimilated carbon (Hellebust, 1965; Nalewajko, 1966; Samuel, Shad and Fogg, 1971; Thomas, 1971).

The potential significance of extracellular organic material in marine food chains is extremely interesting. Many authors (Fogg, 1966; Brock, 1966; Alexander, 1971; Whittaker and Feeney, 1971) have suggested that these products may play an important role in marine food chains, especially as potential nutrients for bacteria. However, to our knowledge, there is no direct evidence that this is so—although the ability of bacteria to grow in algal cultures (Vela and Guerra, 1966; Berland, Bianchi and Maestrini, 1969) might be interpreted to support such conclusions.

If, in fact, algal extracellular products are important contributors to bacterial food chains, it would seem possible to construct an aquatic counterpart of the well-known "rhizosphere" of terrestrial ecosystems (Rovira, 1965). A zone may exist, extending outward from an algal cell or colony for an undefined distance, in which bacterial growth is stimulated by extracellular products of the alga. For purposes of discussion in this paper, we will term this region the "phycosphere."

Motile bacteria commonly exhibit chemotaxis to concentration gradients of organic material (Weibull, 1960; Adler, 1969). The ecology of chemotaxis by organotrophic bacteria has not been well studied, but highly species-specific responses to certain carbohydrates, amino acids, and nucleotide bases have been observed (Fogel, Chet and Mitchell, 1971), and certain predatory microorganisms have been shown to be chemotactic to their prey (Chet, Fogel and Mitchell, 1971).

It is possible that chemotaxis may also be of importance in the establishment of a phycosphere microflora.

In the current studies, we have investigated the validity of the phycosphere concept in a number of ways. We have especially been concerned with the existence and importance of the phycosphere effect during various stages of algal cell growth and death, and the relationship of bacterial chemotaxis to the establishment and maintenance of a phycosphere microflora.

Materials and Methods

Axenic cultures of marine algae were kindly supplied by R. R. L. Guillard, Woods Hole Oceanographic Institution. The following were used in the studies reported here: *Skeletonema costatum* (clone SKEL), *Cyclotella nana* (clone 3H), *Dunaliella tertiolecta* (clone DUN), *Isochrysis galbana* (clone ISO).

The algae were maintained on culture medium F/2A, a slight modification of sea water enrichment medium F/2 (Guilliard and Ryther, 1962), containing 1000 μM $NaNO_3$ and 50 μM K_2HPO_4 per liter. All cultures were grown in 50-ml batches in 125-ml Erlenmeyer flasks at 16° C with 900 foot-candles fluorescent illumination supplemented by four 25 w incandescent bulbs; lights were programmed to an 18-hr-on, 6-hr-off cycle. Cell counts were made using a brightline hemocytometer (American Optical Co., #1492). Cultures were routinely transferred every 10 days.

Algal culture filtrates were obtained by centrifuging aliquots 5 min at 1000 rpm in a table-top clinical centrifuge (International Equipment Co., model CL), followed by sterile filtration through 0.45-μm membrane filters (Millipore HA) which had been pre-washed with a total volume of 10 ml synthetic sea water. Microscopic examination of cells following centrifugation and filtration failed to reveal any detectable cell damage not already in the cultures being sampled. In order to cancel the effects of any organic material introduced into the filtrates from the Millipore filters, F/2A, synthetic sea water, and natural sea water were similarly filtered when used as controls in chemotaxis experiments. All filtrates were stored frozen in screw-capped vials until just before use.

Bacteria were cultured on medium ISOL having the following ingredients per liter: 5.0 g peptone (Difco), 1.0 g yeast extract (Difco), 0.001 g K_2HPO_4 filtered or synthetic sea water to volume. When used for plating, this medium was solidified with 1.5% agar (Difco).

The synthetic sea water medium (SSW) used in these experiments had the following composition per liter: 1.13 g $CaCl_2$, 7.0 g $MgSO_4$, 5.3 g $MgCl_2 \cdot 6H_2O$, 0.72 g KCl, 25 g NaCl, distilled water to volume.

The composition of SSW was chosen to approximate the composition of natural sea water in terms of the most abundant salts. It also satisfies the major ionic requirements of most marine bacteria (MacLeod, 1965). The general suitability of this synthetic sea water is reflected in the fact that all bacterial isolates obtained during these studies could readily be grown on ISOL in which SSW had been substituted for natural sea water. Of course, all bacterial media are selective in one way or another, and the media reported here are no exceptions. Problems introduced by our "selective" media were not evaluated, but they are believed to be minor.

Bacterial isolates were obtained from enrichments of aseptically-obtained sea

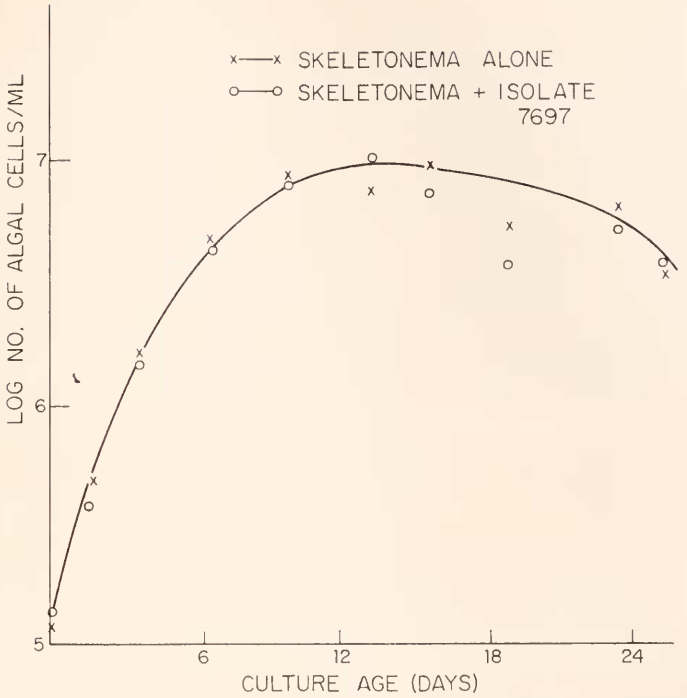

FIGURE 1. Growth of *Skeletonema costatum* in batch culture in the presence and absence of the bacterial isolate 7697. No effect of the bacterium on algal growth was evident in these experiments.

water samples taken from Vineyard Sound near Woods Hole, Massachusetts. The enrichment, begun within 2 hrs after obtaining a sample, consisted of 5 ml of sample + 50 ml of ISOL in 125-ml Erlenmeyer flasks. These were incubated with shaking at room temperature (approximately 27° C) for 18–36 hr before use. These techniques essentially selected for those bacteria able to grow rapidly on a rather rich complex medium. There was no particular selection for specific nutritional types other than for bacteria able to grow aerobically. A more specific technique used to obtain bacteria responding to algal culture filtrates is reported later in this paper. Overnight enrichments always contained a large number of highly motile bacteria.

Chemotactic assays of bacteria, whether obtained directly from mixed enrichments or from pure isolates, were complicated by a variety of behavioral nuances, including the rapid settling of some bacteria onto surfaces and general loss of response upon repeated subculturing. These problems were not completely overcome, but the following procedure yielded the most reliable bacterial preparations: 50-ml cultures were grown on ISOL overnight at room temperature with shaking; 3–6 hr before an experiment, a 2-ml aliquot was inoculated into a second 50 ml of ISOL. After the culture became turbid, the cells were harvested by centrifugation 10 min in a clinical table-top centrifuge (International Equipment Co., model CL)

at 2800 rpm; although the supernatant remained slightly turbid, an appreciable pellet was always obtained. The supernatant was drawn off and the cells resuspended in 5 ml sterile SSW. After a second centrifugation, the cells were resuspended again in sterile SSW to a concentration of 10^6–10^7 per ml. Microscopic examination of such preparations showed that 10–90% of the bacteria were motile; only preparations having a high degree of motility were utilized in experiments.

Bacterial chemotaxis was assayed using the method of Adler (1969). Agar plugs were used to close the 1 μl capillaries. Chemotaxis experiments were run at room temperature for 15 to 45 min. During this time bacterial motility and behavior was monitored visually in selected preparations and controls with a phase contrast microscope. At the end of an experiment the contents of each capillary tube were diluted into 10 ml of sterile SSW and bacterial counts made by plating aliquots on solidified ISOL. Chemotaxis experiments were run in duplicate or triplicate.

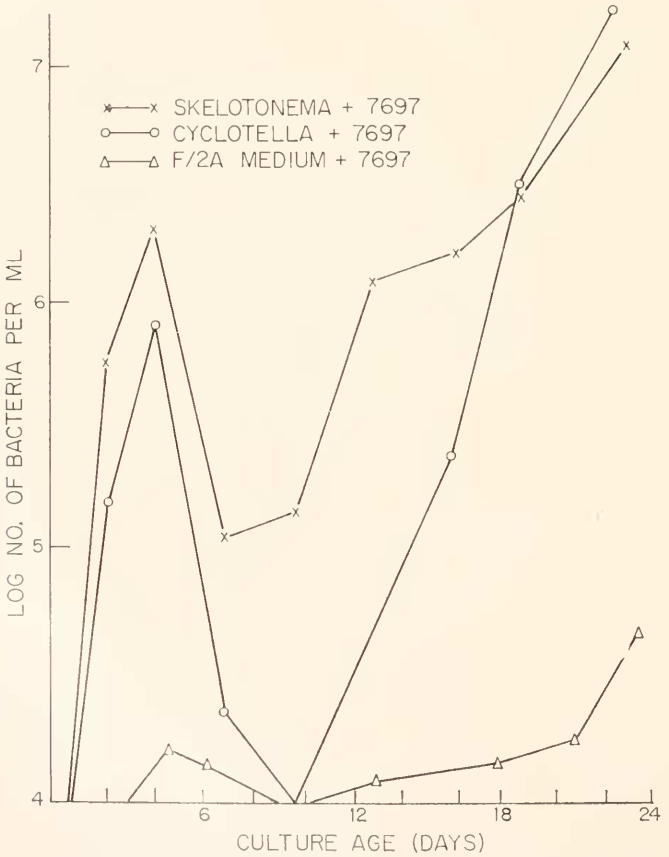

FIGURE 2. Growth of the bacterial isolate 7697 in the presence and absence of two algae, *Skeletonema costatum* and *Cyclotella nana*. The *Skeletonema* culture is the same one depicted in Figure 1. Bacteria were inoculated into these cultures at 0.5×10^4 per ml.

Results

In the first series of experiments, we examined the growth of mixtures of algae and bacteria. These experiments were designed to test for production of either anti-bacterial toxins or bacterial stimulants by the algae. Pure cultures of motile bacterial isolates were washed free of growth medium by centrifugation and resuspension in sterile SSW, then inoculated into freshly transferred axenic algal cultures. Controls consisted of separate algal and bacterial cultures in F/2A medium inoculated at the same time and incubated under the same conditions. The only major carbon source for the bacteria consisted of extracellular material produced by the algae.

Typical results from these experiments are shown in Figures 1 and 2. The two bacterial isolates tested had no discernible effect on algal growth. However,

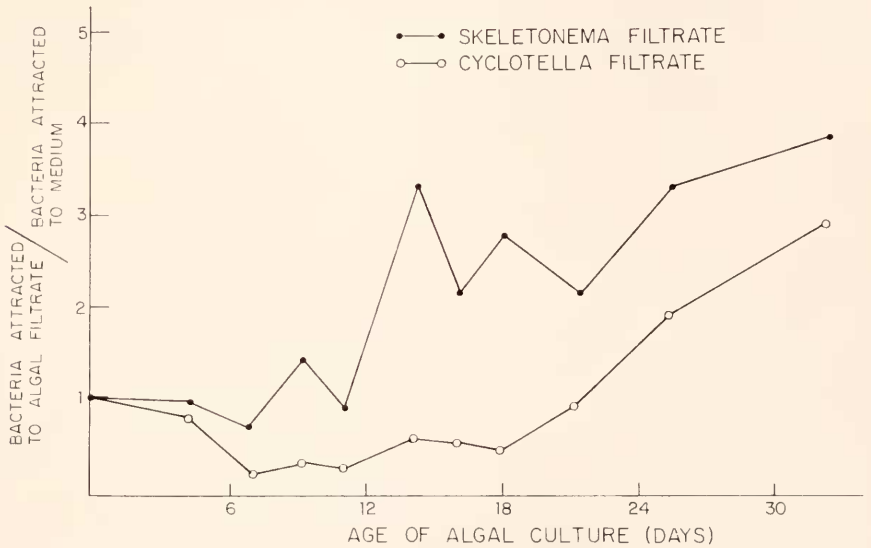

Figure 3. Chemotactic response of bacterial isolate 7697 to filtrates from algal cultures of increasing age. Values on the ordinate are given as the ratio of the number of cells in capillary tubes containing algal culture filtrate to the number in tubes containing F/2A medium. Experiments lasted 45 min. at room temperature.

viable cell counts showed that the bacteria were strongly affected by the presence of algal cells. There was typically an initial burst of bacterial growth during the first week of culture. This was followed by a period of either no increase or a significant decrease in viable cell count, approximately coinciding with the transition period between logarithmic and stationary algal growth. Invariably a marked increase in viable bacterial cells was observed as the cultures aged further.

When compared with the experimental flasks, bacterial growth in the controls was insignificant. The bacterial concentration was always several orders of magnitude less. A slight increase in viable cells during the first week probably represents growth on the organic material present in the natural sea water base of F/2A

medium; the gradual increase with age can be accounted for by evaporation of the medium in the flasks.

It is evident that the bacteria tested are able to coexist in culture with these algae. The data suggest that most of these bacteria depend on degradation products of the algal cells. A small residual population of bacteria existed in a viable state throughout the 30-day period tested, even in control flasks.

In order to study the possible ecological role of these materials further, an assay utilizing bacterial chemotaxis was developed and employed in a series of experiments with intentions similar to those reported above—*i.e.*, the determination of the effect of algal culture age on bacterial response. Controls for these experiments consisted of capillary tubes containing filtered F/2A of the same age as the algal culture being tested.

The results of one such experiment are shown in Figure 3. The bacterium was isolate 7697, one of those used in the previous growth experiments. The chemotaxis pattern obtained is typical and can profitably be compared with the growth data of Figure 2. Filtrates from young algal cultures did not attract the bacterium,

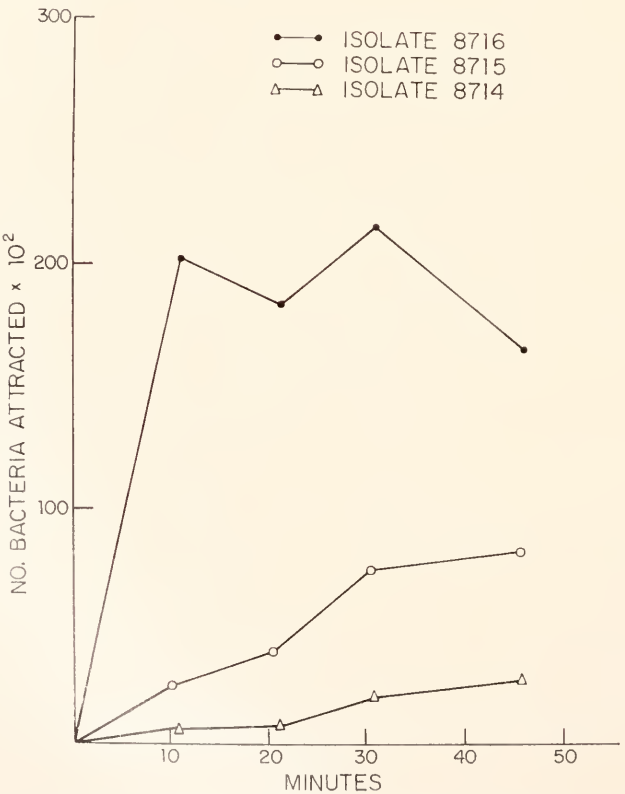

FIGURE 4. Attraction of bacteria from enriched Vineyard Sound water to filtrate from 30-day *Skeletonema* culture. These bacteria, from the same water sample, could be readily differentiated on plates on the basis of colony morphology and color. No other bacteria appeared in significant numbers in this experiment.

but chemotaxis increased markedly when filtrates from older cultures were used as attractants.

Taken together, the data presented in Figures 1–3 strongly suggest the stimulation of a "phycosphere effect" by the algae. However, the data obtained indicate that algal excretions are important only after algae have ceased rapid growth and commenced decomposition.

A series of experiments were initiated to examine the ability of algal culture filtrates to select for specific bacteria from a mixed population. This process is critical in the construction of a phycosphere effect mediated by algal extracellular products. We utilized filtrates from 30-day algal cultures as attractants. These were selected because of their maximum attractiveness to bacteria in previous experiments. Adequate controls were difficult to construct, but Millipore filtered algal medium F/2A of the same age was used as the best compromise. Because bacterial populations in natural sea water are too low to assay quantitatively by this

TABLE I

Attraction of bacterial isolates to filtrates from 30-day algal cultures and to a peptone solution. Data are expressed as for the ordinate in Figure 3; 30-day old uninoculated F/2A medium was used in control tubes. Bacterial response is statistically significant ($P = 0.05$) if the ratio is 2.0 or greater

Algal culture filtrates	Bacterial clone			
	8712	8714	8715	8716
	No. of bacteria attracted/No. attracted to control medium			
Skeletonema	4.2	3.2	2.9	65
Cyclotella	3.4	5.2	—	95
Dunaliella	3.3	3.4	2.1	122
Isochrysis	0.3	3.1	0.8	51
0.5% peptone	7.9	3.3	6.7	46

technique, overnight enrichments of whole water samples were tested. No attempt was made to isolate "dominant" bacteria from the water samples. This is justifiable, since any true phycosphere would also be an enrichment bearing little relation to the dominant bacteria outside the zone of influence (Rovira, 1965).

Figure 4 shows the results of one such experiment using a mixed bacterial enrichment from Vineyard Sound. The three bacterial types indicated could readily be distinguished on the basis of colony appearance on the counting plates. Other bacteria may have been present but were not seen at the dilutions counted. These data indicate that algal culture filtrates are indeed capable of eliciting chemotactic responses from a non-specific enrichment, and presumably from indigenous bacteria in the water column, with the degree of response differing between bacterial types. Such observations can be exploited in the laboratory for the purpose of obtaining bacteria that respond to specific algal species. Because these experiments utilized a mixed bacterial system, however, caution must be used in labelling bacteria as "strong" or "weak" in their response, as the presence of other bacteria may lead to undefinable interactions.

During the visual monitoring of this particular experiment, variations in bacterial behavior could be readily discerned and later correlated with the specific isolates obtained. The *Spirillum*, isolated as clone 8716, entered the capillary tube extremely rapidly and within 15 min could be found along the entire length. Such behavior is characteristic of spirilla and is often utilized in their isolation (Veldkamp, 1970). The small pseudomonad, isolated as clone 8715, entered less rapidly but by the end of the experiment it had formed a band of high concentration just inside the mouth of the tube. Isolate 8714 was at too low a concentration in the tube to be studied visually.

After their isolation, bacterial strains were tested separately for their ability to respond to 30-day algal culture filtrates and compared with their response to 0.5% peptone, a rather rich organic attractant (Table I). In general, the isolates responded to the culture filtrates as well as, or better than, peptone, with the exception of *Isochrysis* filtrate. The best response was shown by isolate 8716, paralleling its behavior in the mixed enrichment. Despite the possibility of interspecific bacterial interactions in the mixed enrchments, the data of Table I confirm that the bacterial responses are independent of other bacteria.

From the above data it can be concluded that specific bacteria may be selected from a non-specific mixture by algal extracellular products. This selection would be mediated by the chemotactic responses of the bacteria. Subsequent experiments were designed to evaluate this possibility to see if the laboratory studies could be extrapolated into natural systems.

TABLE II

Survey of bacterial chemotactic responses to chemicals identified in filtrates from marine algal cultures. Data expressed as for ordinate in Figure 3, with SSW serving as a control The response is significant ($P = 0.05$) if the ratio is greater than 2.0. All chemicals were tested at a concentration of 10^{-2} M in SSW

Attractant	Bacterial clone			
	8712	8714	8715	8716
	No. of bacteria attracted/no. attracted to control mediu			
Amino acids:				
alanine	4.3	1.3	7.2	24
valine	5.3	0.7	5.3	24
proline	1.7	1.3	4.6	9.5
lysine	2.1	0.4	3.4	146
arginine	8.9	1.4	5.6	87
methionine	8.6	2.5	4.1	—
glutamic acid	0.2	0.0	0.1	0.0
aspartic acid	0.0	0.0	0.0	0.0
Polyalcohols:				
mannitol	0.8	1.3	1.8	43
glycerol	—	1.5	1.5	1.0
Sugars:				
glucose	1.1	1.8	1.0	1.7
sucrose	1.3	4.9	2.2	39

The construction of adequate control experiments is difficult because of the labile nature of algal culture media. These can change their properties with age whether algae are present or not (Provasoli, McLaughlin and Droop, 1957). We did not detect any significant change in any of our media during thirty days of storage. F/2A was therefore used routinely when testing for chemotaxis to algal culture filtrates. However, SSW, expected to have a lower background concentration of organic material, was employed when testing for chemotaxis to specific chemicals.

Hellebust (1965) reported on the nature of extracellular products from many marine phytoplankters, including those algae studied here. These compounds included simple amino acids, sugars, and polyalcohols; extracellular peptides and polysaccharides were also implicated by the increase in amino acids and monosaccharides after acid hydrolysis of culture filtrates.

If extracellular products are to be implicated as bacterial attractants or related in any other way to the establishment of a phycosphere microflora, the compounds identified by Hellebust (1965) would be expected to be among the active components.

Several of the fresh bacterial isolates shown to be chemotactic to algal culture filtrates were tested for their chemotactic response to Hellebust's (1965) extracellular products. The results (Table II) show that the amino acids elicited the best responses. Curiously, the common metabolite glucose was not a good attractant, though most bacteria responded to sucrose. Glycerol and mannitol, identified in extracellular products by Hellebust (1965), were generally not atractive. The response to glutamic and aspartic acids was low, due to inhibition of bacterial motility around the mouth of the capillary tube. When these compounds were tested dissolved in sea water, there was no chemotactic response although motility was not inhibited.

The absolute concentrations of specific compounds among the extracellular products of marine algal cultures is technically difficult to determine because of problems associated with desalting the medium prior to concentrating the organic material. Some preliminary experiments in this laboratory, studying the extracellular production by the marine alga *Chlorella* sp. (Woods Hole clone 580) indicate concentrations of 10^{-8}–10^{-6} M for amino acids and sugars found in the filtrates of log phase cultures.

Experiments to determine the threshold for bacterial chemotactic response were constructed to compare with such information. These experiments consisted of chemotaxis assays of single bacterial preparations using increasing tenfold dilutions of selected organic compounds in SSW. In almost all cases, the values were found to lie between attractant concentrations of 10^{-6}–10^{-5} M. This range agrees well with threshold concentrations found for chemotactic responses of *Escherichia coli* to monosaccharides as reported by Adler (1969). Considering the very low concentrations of organic material found in algal culture material and likely to be found in natural waters, these thresholds are surprisingly high.

Discussion

The construction of a theory to account for the development of a phycosphere is dependent on two criteria. There must be a source of enrichment for the microbial population in proximity to the algae. The microflora must respond to the

algal products by being attracted and/or growing in this region. The data presented will be discussed from the point of view of these criteria.

The chemical nature of algal extracellular products renders them likely sources of microbial nutrients. As they may constitute a significant portion of primary production, such compounds are indeed of potential significance in microbial food chains. There is considerable confusion in the literature, however, as to the source of these compounds. Short-term experiments such as the ones of Fogg, Nalewajko and Watt (1965) and Watt and Fogg (1966) strongly suggest that the materials may be released as products of cell metabolism, a process sometimes termed "excretion." On the other hand, long-term experiments lasting several days such as those of Marker (1965) have shown that the increase in soluble organic carbon in algal culture filtrates might readily be attributed to cell lysis, on the order of 1 in 100–1000 cells daily. Accurate determination of cell lysis by counting techniques has so far not proved technically feasible.

This difficulty in determining the actual source of extracellular organic material under natural conditions complicates ecological interpretation of the effect of this material on bacterial populations. In our study (Figs. 1 and 2) we were able to demonstrate that bacteria were indeed able to grow in algal cultures with no additional carbon source—an observation known to all workers who routinely isolate algal cultures and by no means a novel one (Berland, Bianchi and Maestrini, 1969).

The data contain two additional important observations, however. There was no discernable predation of bacteria on the algae. This reinforces the conclusion that the material on which the bacteria were growing was indeed extracellular. In addition, bacterial growth was maximal during the declining stage of the algal growth curve, when algal cell lysis was evident. It was often possible to observe the presence of bacterial aggregates around clumps of lysed algal cells.

The observations shown in Figure 2 also include an increase in bacterial concentration during the early stage of algal growth. The source of organic material for this increase has not been determined, although it probably was extracellular. It appears, however, that algal extracellular products may have the greatest impact on the bacterial community only during the latter stages of a phytoplankton bloom, when algal cell lysis is highest.

The second criterion for the establishment of a phycosphere, bacterial response, was studied in more depth. The data show conclusively that bacteria are capable of growing in algal cultures. The behavioral response, in this case chemotaxis, was studied from the belief that if a phycosphere were ecologically significant, motile bacteria might be attracted to this region before commencing growth on the organic material.

Our data indicate that marine bacteria are chemotactic to algal culture filtrates. This response was invariably highest to filtrates from old algal cultures (Fig. 3), implying that the release of extracellular carbon is most important ecologically during the later stages of a plankton bloom. There was no significant chemotactic response to filtrates from younger cultures, even though such cultures supported bacterial growth.

The threshold concentrations of some of the compounds eliciting bacterial chemotaxis were found to lie generally in the range 10^{-5}–10^{-4} M. Nearshore waters

and estuaries usually average 10^{-6}–10^{-5} M for carbohydrate and 10^{-8}–10^{-7} M for specific amino acids (Wagner, 1969). Bacteria were not attracted to natural sea water (compared against SSW) in our experiments. Utilizing bottles incubated *in situ* in freshwater lakes, Fogg and Watt (1965) found that *Chlorella pyrenoidosa* produced a maximum concentration of extracellular glycollic acid of 1.5 mg/l, or about 10^{-5} M. In our own laboratory, the concentrations of extracellular amino acids and sugars in algal cultures are at best an order of magnitude less during logarithmic growth. In both cases, such experiments have utilized closed systems in which the extra cellular products could accumulate unnaturally. Thus, relative to the general concentrations of organics found in nature, the thresholds for bacterial chemotaxis are very high.

These data do not support the second criterion for a phycosphere as far as rapidly growing planktonic algae are concerned. No bacterial response to young algal culture filtrates was observed. While the possibility of bacterial inhibition cannot be eliminated completely, observations of bacterial behavior during such experiments failed to reveal any noticeable inhibition of bacterial motility or general activity. The interpretation most consistent with these data is that the concentrations of extracellular compounds in filtrates from young algal cultures simply were not above the required thresholds for bacterial attraction. In the filtrates from older algal cultures, it would appear that the second criterion for the creation of a phycosphere is met, at least in terms of bactrial chemotactic response to these filtrates.

Bacterial chemotaxis probably serves to keep a bacterial cell near a source of organic material once it has arrived there by chance. A decomposing algal cell thus serves as a bacterial sink. The shock reaction behavior does not aid a bacterium in locating such a sink, but keeps it there once the bacterium gets close enough to respond to the chemical gradient. This effect may be further enhanced by the tendency of motile bacteria to settle onto nearby surfaces rapidly after they begin exhibiting shock reactions in response to a supra-threshold concentration of chemicals. This kind of behavior is very similar to that observed in photosynthetic bacteria responding to a restricted zone of illumination (Pfennig, 1967). Such behavior would help explain the ability of marine bacteria to exist in what is otherwise—in terms of dissolved organic material—a nutritionally poor environment (Jannasch, 1967).

The notion that algal extracellular products must be highly significant in bacterial food chains is far too general to be of much predictive use. The data presented here indicate that a more narrow definition of "extracellular products" is in order, as the bacterial growth and chemotactic response varies greatly with the age of algal cultures and these are, in fact, greatest when algal cells are lysing in old cultures. The role of extracellular compounds released by rapidly growing algae remains to be evaluated, but the general statement that they have a great significance in microbial ecology is totally unwarranted unless qualified by considering highly specific classes of compounds such as antibiotics, vitamins, toxins, *etc.*

We have found that the term "phycosphere" can be a useful one in discussing and evaluating algal-bacterial interrelationships. The phycosphere effect would be expected to be greatest during algal bloom decomposition. This effect is medi-

ated in part by bacterial chemotaxis to organic material released by the lysing algal cells, which serves to keep bacteria in proximity to the cells until most of the available organic material has been utilized. It would appear that the phycosphere is a region of interactions that have only begun to be evaluated.

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Summary

1. The possibility that planktonic algae possess a "phycosphere," a zone surrounding them created by the production of extracellular products which may serve as bacterial nutrients, is examined.

2. Bacterial growth in algal cultures to which no additional organic material is added is greatest only as the cultures age and algal cell lysis becomes obvious.

3. Marine bacterial isolates are chemotactic to filtrates from algal cultures, but the response is significant only to filtrates from old cultures, again where cell lysis is evident.

4. Specific compounds known to occur as algal extracellular products attract bacteria, but the threshold concentrations for attraction are unexpectedly high when compared with the generally very low concentrations of organic compounds in natural sea water.

5. The validity of the phycosphere concept and its potential importance to marine microorganisms is discussed.

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